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The utinga virus: antigenic characterization and serologic epidemiology

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ANTIGENIC CHARACTERIZATION AND SEROLOGIC EPIDEMIOLOGY

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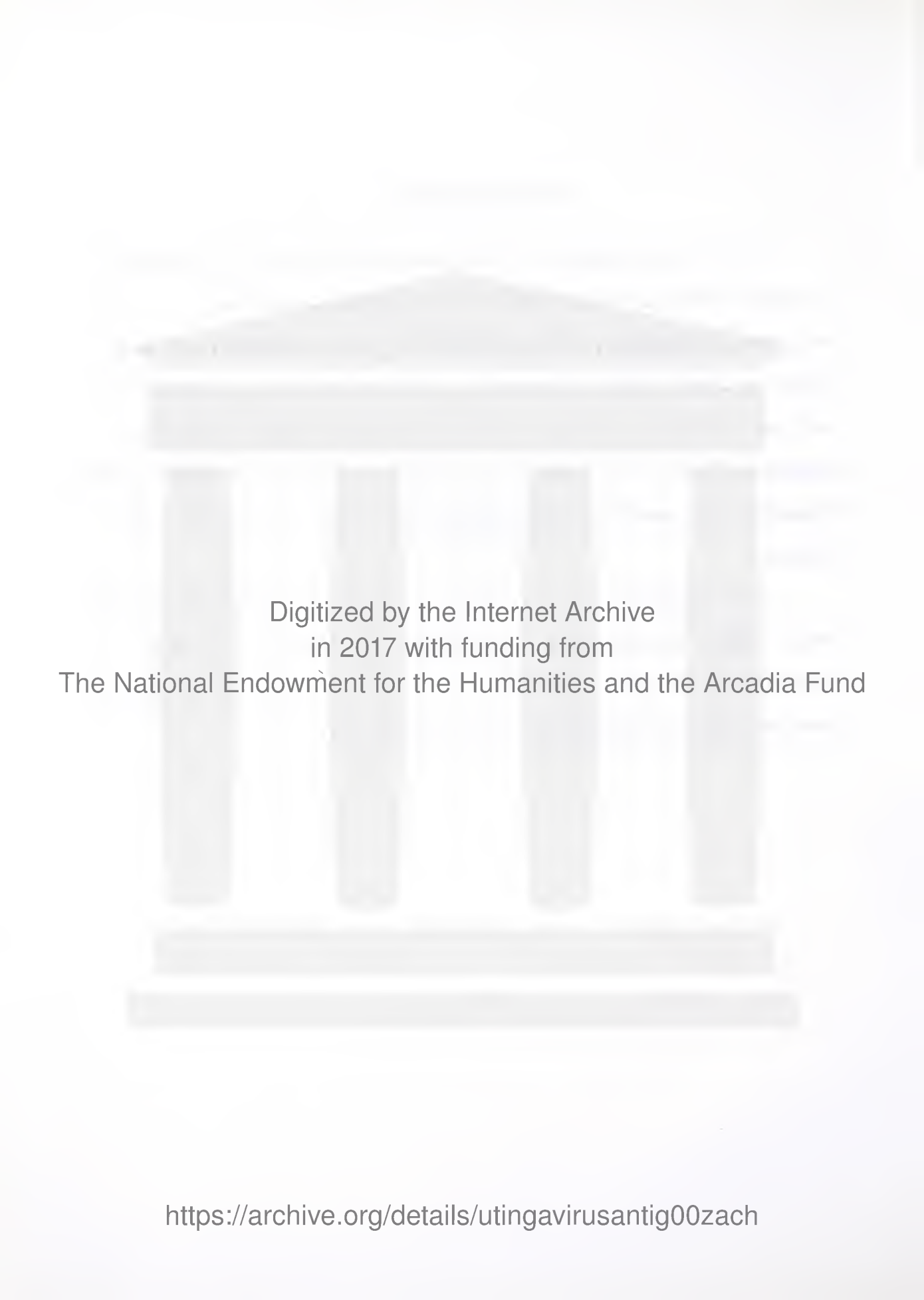

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INTRODUCTION

The Utinga Virus

In October of 1965 a virus was isolated from the blood of the three-toed sloth (*Bradypus tridactilus*) captured in the Utinga Forest near Belem. Serological testing of this virus (BE An 84785) against group specific immune sera of all presently known viruses of that region showed that this agent was serologically related to the Simbu group of arboviruses, but was not identical to any of the known members of this group.[42] The serological relationship of Utinga virus to Oropouche virus of the Simbu group was especially interesting, since Oropouche had been associated with a large epidemic in Belem during the Spring of 1961. Oropouche virus was isolated from 14 febrile cases, and of the patients studied, 42 of 87 converted from negative to positive during the epidemic with respect to neutralizing antibodies.[22] The Oropouche virus had been isolated from man for the first time in Trinidad, a few years prior to the epidemic in Belem.[2,37]

When the newly isolated agent from the Utinga Forest (subsequently named the Utinga virus) was noted to be related to Oropouche virus, it became interesting to speculate whether it could have played any role in the Oropouche epidemic.

The study as presented in this paper was undertaken with three main objectives in mind: (1) to characterize this new agent, the Utinga virus, with respect to its serological reactivity and its relationship to the other members of the Simbu group of Arboviruses; (2) to obtain some idea of the serologic epidemiology of this virus and its prevalence; and (3) to find out its possible association with the Oropouche virus, whether humans infected with Oropouche during an epidemic also develop Utinga antibody, and whether it may have played any role in human illnesses in the Belem area over the last few years.

The Arboviruses - General Consideration

The arboviruses,* arthropod-borne animal viruses, are a large heterogeneous group of viral agents linked together in classification by their capacity to infect certain vertebrates - mammals, reptiles and birds - and to multiply in the body of arthropods without producing, with rare exceptions, any pathological changes in these infected vectors. Such agents are maintained in nature by a continuous cycle in which the arthropod vector becomes infected, generally by ingesting blood from a vertebrate host at a time when the virus circulated in the latter, and, after a period of days, designated as the extrinsic incubation, the vector, by biting can transmit the disease to a new susceptible host.[5,19]

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* officially accepted nomenclature by the subcommittee on virus nomenclature at the International Congress of Microbiology, Montreal, August 1962.

Besides the common biological cycle, certain other characteristics are probably common to most of these agents: (1) an essential lipid envelope making the particle rather highly sensitive to ether and sodium desoxycholate,[19,36] and (2) an RNA core. Also, they are in general rather unstable viruses, readily inactivated by relatively little heat (37-57°C), and all produce encephalitis in suckling mice following intracerebral inoculation.[5,19]

To the present time about 220 different arboviruses have been isolated in various parts of the world and the list is expanding yearly. Through the use of serological tests - neutralization (NT), complement-fixation (CF), and hemagglutination-inhibition (HI)- most of these viruses have now been classified into 22 antigenic groups, principally by Casals and co-workers.[5,6,7,8,9,10,11,40] Some of these groups also contain subgroups of viruses showing very close relationship with each other.

Although many laboratories and workers around the world have contributed to the ever growing list of arbovirus isolates, and to the elucidation of the epidemiology of these agents, there are several laboratories which have been exceptionally productive in this respect. One of these laboratories is the Belem Virus Laboratory (Belem, Brazil) a field laboratory maintained by the Rockefeller Foundation and the Brazilian government since 1954, which has been responsible for the isolation of at least 35 new agents and many already known agents.[12,13,14,30] The

functioning of this laboratory is explained in detail by Causey et al.[12] Over the years this laboratory has accumulated a vast number of sera (human, mammal, bird and reptile) which provide a wealth of material for retrospective serological studies.

Belem - The Study Area

Belem and the surrounding area involved in the study, as shown in Figure 1, are situated on the right bank of the Para River, extending north from the Guama River to the sea, and about 50 km inland. In this area the climate is tropical rain forest, and is characterized by relatively high levels of temperature and rainfall, with minimal annual variation. The mean monthly temperatures varies less than 5°C during the year, and the driest month has at least 6.0 cm. of rainfall.[12]

East of Belem, between 5 and 20 km., is a continuous tract of forest along the Guama River. This is divided into the Oriboca virgin forest with many swamps and streams, the property of the Instituto Agronomico do Norte (I.A.N.), the site of an old cocoa plantation, now reverted to dense second growth, and the Utinga forest, a watershed for the city reservoirs with several large, deep lakes.

The Simbu Group

At the present time 11 different viruses are known in the Simbu group of arboviruses, and most of these have been

isolated only within the last 10 years in various parts of the world. Although antibodies in humans have been detected for Manzanilla, Simbu, and Oropouche viruses, of all the Simbu group viruses only the Oropouche has been shown to cause any human disease, and this agent is also the only one, other than the Utinga virus, that has been isolated in the Belem area. The other members of this group, with the dates and location of isolation, and the species from which isolated are listed in Table I.

MATERIALS AND METHODS

VIRUSES

The following Simbu group viruses were used in testing:

Virus	Strain	Passage level
Akabane	Ja Ar 39	18
Buttonwillow	A 7956	5
Ingwavuma	SA An 4165	7
Manzanilla	TRVL 3587	4
Oropouche	TRVL 9760	5
Sathuperi	IG 11155	3
Simbu	SA Ar 53	12
Utinga	BE An 84785	3
Sango	Ib An 5077	5
Ib An 5550	-	3
Yaba 7	-	5

HEMAGGLUTINATION-INHIBITION TESTS

The major part of the serological survey was done employing the hemagglutination-inhibition (HI) test. The technique used was that described by Clarke and Casals[15] with some modifications.

Preparation of Hemagglutinating Antigens

The HA antigens were prepared from the brains of 2-4 day old mice inoculated by the intracerebral route with 0.02 ml. of a 10^{-2} dilution of stock infectious suckling-mouse brains. The mice were harvested (on the eighth day in the case of Utinga virus) when some of the mice began to die, and many others appeared sick from the infection. The harvested mice were kept in polyethylene bags at -70°C until ready for use.

The HA antigen was prepared by the sucrose-acetone extraction method.[15] This procedure has been used increasingly

over the last few years at the Rockefeller Laboratories and is proving to be superior to all other methods.

The removed and weighed suckling-mouse brains were homogenized with four volumes of a chilled 8.5% aqueous solution of sucrose, and the homogenate added, by squirting from a syringe, to twenty volumes of chilled acetone. This mixture was shaken vigorously, and the supernatant decanted. The same volume of chilled acetone was added to each bottle, and the preparation was allowed to stand in an ice bath for one hour. The supernatant was then discarded and the sediment dried in a vacuum apparatus. The dry powder was rehydrated with 0.85% saline, the volume used being equal to 0.4 of the total volume of homogenate used, and the preparation was left overnight to rehydrate. It was then centrifuged for 30 minutes at 10,000 rpm. The supernatant was distributed to ampoules (0.5 ml), lyophilized, and the ampoules sealed and kept at 4°C until ready for use. About 90 ampoules of Utinga antigen were transported, without refrigeration during a period of about two weeks, to Brazil. Hemagglutination testing upon arrival in Brazil demonstrated that in spite of the prolonged exposure of the antigen to ambient temperatures, there was no loss of titer. In Brazil, the antigen was kept at 4°C without loss of titer over 3½ months.

Sera and Ascitic Fluids

Most of the mammalian sera examined in the survey were obtained from the mammals captured in the Utinga forest,

and most of the bird sera from birds from the I.A.N. area. The human sera from 1960-61 were collected from inhabitants of several districts of Belem during a serological survey for antibodies to the Oropouche virus. The human sera from all subsequent years were collected from cases of fevers of unknown origin (FUO) which presented themselves to the laboratory; these were in many cases employees of the laboratory and their families. Some of these cases were forest workers, many of whom also lived in the forest.

All the sera tested had been stored at the Belem Virus Laboratory for various lengths of time (a few days to several years) at -20°C or -70°C.

Reference immune sera and mouse ascitic fluids were made by a series of 3 to 5 weekly intraperitoneal inoculations in adult mice of 0.1 ml of infected suckling-mouse-brain suspension prepared in saline and Freund's complete adjuvant (Difco Laboratories).

After several i.p. inoculations with the antigen, the mice were inoculated with 0.1 ml of ascitic fluid from mice with ascites secondary to Sarcoma 180/TG. When these mice developed ascites (10-14 days) the ascitic fluid was removed using a 50 cc syringe with an 18 G needle. Mice that survived this procedure, were paracentesed again at intervals of a few days. The ascitic fluid was centrifuged for 10 minutes at 10,000 rpm to remove tissue debris, and the supernatant fluid was used as reference immune ascitic fluid in all subsequent HI, CF, and NT tests.

The Sarcoma 180/TG is a slow-growing strain of the tumor which was originally selected for resistance to the synergistic antineoplastic drug combination azaserine and 6-chloropurine.[27] Since it develops more slowly in mice, and is much less invasive than the original Sarcoma 180, as used by Herrmann and Engle[17] and Tikasingh et al,[38] considerably larger volumes of ascitic fluid can be obtained. The immune ascitic fluids thus produced have been shown to have antibody titers which compare very favorably with those of the sera from the same mice, and to be of high specificity.[27]

Preparation of Sera for HI Test

All sera were treated by the acetone extraction method of Clarke and Casals[15] to remove the nonspecific inhibitors of arbovirus hemagglutinins, which, as observed by Porterfield and Rowe[24], many sera contain.

Routinely 0.05 ml of serum was treated for all HI tests. The serum was diluted 1:10 in 0.85% saline. Using a dropper pipette with a #23 disposable needle the serum was squirted into 12 volumes chilled acetone; the preparation was shaken and allowed to stand in an ice bath for five minutes. The tubes were then centrifuged one minute at 1,000 rpm, the supernatant decanted, and the precipitate resuspended by shaking. An additional 12 volumes of acetone was added and the preparation was allowed to stand for one hour in an ice bath. The tubes were then

centrifuged 5-8 minutes at 1,500 rpm, the supernatant decanted, and the precipitate in the tubes was dried for one hour in a vacuum jar at room temperature. The dry powder was rehydrated with 0.5 ml. (10 volumes with respect to the original volume of serum) of borate-saline pH 9.0 solution. This gave a final serum dilution of 1:10 based on the volume of serum initially introduced.

Since it has been shown by Porterfield[23] that many sera contain naturally occurring agglutinins for goose erythrocytes, all sera were routinely absorbed with such cells before being used in the tests. The goose erythrocytes were obtained by bleeding geese kept at the laboratory, with the blood collected in 50 cc syringes with acid-citrate-dextrose (ACD). The blood was then centrifuged for 10 minutes at 1,000 rpm, the plasma aspirated off by a 10 cc pipette, and the cells then washed three times with saline, each time followed by centrifugation for 10 minutes at 1,500 rpm. The final volume of packed cells was diluted with 5 volumes of 0.4% Bovine-albumin in borate-saline (final dilution 1:6).

To each serum (0.5 ml) was added 0.6 ml of the 1:6 dilution of goose erythrocytes. This preparation was allowed to stand for 20 minutes in an ice bath with occasional shaking. The tubes were then centrifuged for 15 minutes at 1,500 rpm, the supernatant decanted, and used for the HI tests, the serum at this point being at a 1:20 dilution.

The packed washed goose erythrocytes were also used for preparing the cell suspension used in the HI test proper. The packed cells were diluted 1:8 with dextrose-gelatin-veronal (DGV) and kept as such as a stock suspension of cells. By testing the hemagglutinating antigen of the Utinga virus at various pH's from 6.0 to 7.0 it was found that the antigen showed optimum agglutination at pH of approximately 6.4, and therefore this pH was used in all subsequent HA and HI tests. The virus-adjusting-diluent (VAD) solution of the desired pH (or approximately 6.4) was prepared by combining phosphate buffer solutions of pH 6.0 and pH 7.0 in the proportions of 6:4. Goose cells were added to the VAD solution in the proportion of one part cells (at 1:8) per 39 parts diluent at the desired pH. This final preparation was used for the HA and HI tests.

Methods for the HA and HI Tests

All the HA and HI tests were carried out using the microtechnic which is based on the modification by Sever[28] of a microtitration method originally introduced by Takatsy. This micro method has the advantage of facility of performance and conservation of reagents and sera. It employs small plastic plates with rows of wells (see Figure 2) in which the reactions are carried out, and calibrated wire loops for making the dilutions.

Preliminary tests using both the older macro method of tube dilutions, and the micro method with loop dilutions confirmed the findings by Sever[28] and Shope[29] that these methods have comparable sensitivities and accuracy.

All the sera were tested in 1 or 2 dilutions against one dilution of antigen adjusted to give about 4 units of hemagglutinin per 0.1 ml. The Utinga virus antigen used gave 4 units of hemagglutinin at a dilution of 1:4 and this was the dilution used for most of the tests. The number of units of hemagglutinin is calculated by considering the highest dilution of antigen which gives complete or nearly complete agglutination as giving one unit of hemagglutinin per given volume.

All dilutions of sera and antigen were made in bovine-albumin-borate-saline pH 9.0 solution.

All components of the test were distributed on the plates using a dropper pipette with a blunt 18 gauge needle calibrated to give 0.025 ml per drop.

The acetone treated sera at 1:20 dilution were distributed in the wells, and one 2-fold dilution made with the wire loops (1:40 dilution). One control well was used for each serum to rule out any non-specific goose cell agglutinins in the sera. Diluted antigen, giving 4 units of hemagglutinin per 0.025 ml was then distributed to all the wells except the controls.

With each test a titration of the antigen was done starting with the dilution of the antigen as used for the

test. A control of the diluent was also done with each test, to exclude the possibility of non-specific agglutination by the diluent. The plates were then incubated overnight at 4°C.

The next day the goose erythrocyte suspensions were prepared at pH 6.4 in VAD solution, as explained above, and 2 drops (0.05 ml) of the cells were added to all the wells on the plates. The plates were gently shaken by tapping, incubated at 37°C for 45 minutes and read. The plates were read as + = complete hemagglutination (no hemagglutination-inhibition); + = partial hemagglutination (trace hemagglutination-inhibition); ± = trace hemagglutination (partial hemagglutination-inhibition); 0 = no hemagglutination (complete hemagglutination-inhibition). Only sera showing complete inhibition were considered positive. All sera giving complete or partial hemagglutination-inhibition were retested using six serial 2-fold dilutions of sera: 1:20 - 1:640.

NEUTRALIZATION TESTS

Neutralization tests in mice were carried out to substantiate the findings of the HI tests. Cross-neutralization tests in mice were performed to further elucidate the relationships of the Utinga virus to the other members of the Simbu group.

The neutralization test is based on the principle that when a specific immune serum is added to its corresponding virus, the virus is rendered noninfective or is "neutralized."

While various animals are used for neutralization tests, the Swiss mouse has been found to be a suitable animal for work with arboviruses. This animal has the advantage both from the standpoint of cost, since for these tests large numbers of animals have to be used, and from the standpoint of host susceptibility, since it has been shown that all currently known arboviruses produce infection and disease in suckling mice when inoculated by the intracerebral (IC) route.[5,19] This susceptibility was first demonstrated with yellow fever virus by Theiler in 1930.[35]

The methods used for the present study were those as employed currently at the Yale Arbovirus Research Unit and are outlined briefly below.

The albino Swiss mice, Charles River strain, were used exclusively for all tests. Since some agents are pathogenic only for the very immature mouse, even by the intracerebral route, and since the Utinga virus appears to have a fairly long incubation time (about 6-10 days), only 2-4 day old mice were used. All the baby mice used for any one test were pooled together in one box, carefully mixed, and distributed back randomly to the individual cages, eight mice per cage.

The constant serum-varying virus method was employed for all tests. This method has the advantage that it has been more frequently used <u>in vivo</u>, more is known about its variables, the end points of titration are rather well defined, and it is readily subject to standardization

for reference to any constant or index, such as the neutralization index.

The viruses employed were 10% infectious suckling-mice brain in 0.75% Bovine-albumin-phosphate pH 7.2 (BAP). Two types of virus preparations were used. For some of the tests a lyophilized preparation of the above was used which had been kept in sealed ampoules at -20°C, and was rehydrated just prior to use with the appropriate amount (0.5 or 1.0 ml) of 0.75% Bovine-albumine-phosphate pH 7.2. Some of the tests were done using a 10% brain in BAP not lyophilized, but kept in sealed ampoules at -70°C. Just prior to use the 10% infectious virus brain preparation was rehydrated with 0.75% BAP, or simply thawed out if the frozen material was used, and serial 10-fold dilutions made in tubes.

All the mouse immune ascitic fluids (prepared as described previously) which were used for the cross-neutralization tests were stored in lyophilized form in sealed ampoules at -20°C. The only exceptions to this were the immune ascitic fluid to the Utinga virus and the normal ascitic fluid used for controls. These were stored frozen without lyophilization in plastic tubes at -20°C. The sera used in the neutralization tests done in Belem, and the mouse immune ascitic fluid to the Utinga virus used as control in these tests, were stored frozen at -20°C.

Just prior to use, the lyophilized ascitic fluids were rehydrated with 0.75% BAP, and the frozen ascitic

fluids or sera thawed out at room temperature. The ascitic fluids or sera were then distributed by pipette to the appropriate tubes. An equal volume of the appropriate virus dilution was added from the master tubes, and the tubes were incubated in a water bath for 60 minutes at 37°C. During the time of inoculation the tubes were kept in an ice bath. No fresh animal serum (i.e., accessory factor) was used. Ascitic fluids were not inactivated.

Suckling mice (2-4 days old) were inoculated intracerebrally with 0.02 ml using 0.25 cc glass tuberculin syringes with 26 gauge needles. One cage (8 mice) was inoculated with each dilution. The mice were checked daily for incidence of illness or death and dead mice were discarded. A daily record of morbidity and mortality was kept on standard "mouse cards." (See Figure 3.)

Calculation of the Neutralization Index

The results of the neutralization and cross-neutralization tests were calculated on the basis of a 50 per cent end point expressing a 50 per cent mortality or LD_{50} (50 percent lethal dose). The method of Reed and Muench[25] was used for calculating the LD_{50} and the neutralization index. Although this method is applicable primarily to a complete titration series, that is, the whole reaction range, from 0 per cent to 100 per cent mortality, it can be used even when these conditions are not fulfilled, if the reactions occur in a uniform manner over the range of dilutions employed.

A hypothetical arrangement of data used in computing the LD_{50} titer by the Reed-Muench formula is as follows:

Virus Dilution	Died	Survived	Accumulated Values: Died	Survived	Mortality Ratio	Mortality Per Cent
10-1	8	0	22	0	22/22	100
10-2	6	2	14	2	14/16	88
10-3	5	3	8	5	8/13	62
10-4	3	5	3	10	3/13	23
10-5	0	8	0	18	0/18	0

The necessary proportionate distance of the 50 per cent mortality end point is obtained as follows:

$$\frac{(\%\text{ mortality at dilution next above } 50\% - (50\%)}{(\%\text{ mortality at dilution next above } 50\% - (\%\text{ mortality at dilution next below } 50\%)} = \text{Proportionate Distance}$$

For the above example the log LD_{50} titer = 3.3

The log neutralization index, in which form the results are presented, indicates the capacity of the serum to neutralize the action of the virus on the host system employed. It represents the difference between the titer of the virus in the presence of normal control serum and the titer of the virus in the presence of the test serum, and is obtained by subtracting the logarithm of the LD_{50} titer of the immune (or test) serum from the logarithm of the LD_{50} titer of normal control serum.

COMPLEMENT-FIXATION TESTS

The procedure used for the complement fixation test was that employed presently at the Yale Arbovirus Research Unit (YARU) and is based on the method described by Casals[4]

with the later modifications by Fulton and Dumbell[16] and by the Rockefeller laboratory. It is a micro method employing plastic plates with rows of small wells similar to the plates used for the HI tests (see Figure 2), and thus has the advantage of conservation of reagents.

Complement Titration

Prior to each test the complement was titrated using the test tube method (100x13 mm tubes), and again in the test itself on the microplates, with the results always in close agreement by both these methods.

Veronal buffer, pH 7.4, was used for making all the dilutions in the test and for controls. In the CF tests done at YARU a commercial guinea pig complement was used (Baltimore Biological Laboratory, Baltimore, Maryland), while in Brazil, frozen pooled guinea pig serum was used as the source of complement.

Fresh complement at a dilution of 1:30, was prepared just prior to each test and nine serial dilutions made in the following manner:

	1	2	3	4	5	6	7	8	9
c'(1:30)ml.	0.1	0.2	0.3	0.35	0.4	0.5	0.6	0.7	0.8
Veronal diluent ml.	1.9	1.8	1.1	1.65	1.6	1.5	1.4	1.3	1.2

Sensitized sheep erythrocytes were prepared by combining equal volumes of 4% sheep erythrocytes (provided as either a commercial 10% sheep cell suspension from Baltimore Biological Laboratory or as 10% erythrocytes from sheep kept at the laboratory at Belem) with an equal

volume of anti-sheep hemolysin at 1:800 (Baltimore Biological Laboratory, Inc. West Chester, Penn.). The hemolysin-cell suspension was then incubated for 15 minutes in a water bath at 37°C. The sensitized sheep cells were prepared in the same manner for both the complement titration and for the complement fixation test.

0.1 ml. of the sensitized sheep cells was added to nine tubes containing 0.2 ml of the serially diluted complement and 0.1 ml. of veronal diluent. The tubes were incubated for 30 minutes in a 37° C water bath, placed in the refrigerator at 4°C for about one hour to allow for complete settling in the tubes, and read on a scale of 0 (complete hemolysis) to 4 (no hemolysis). On the basis of the preliminary complement titration the 1:30 complement solution was adjusted to contain 2 units of c' per 0.1 ml of 1:30 c'. In practice this adjustment was rarely found necessary since the 1:30 c' as prepared, was usually of the exact concentration desired.

Method for the CF Tests

For the CF test each serum or ascitic fluid was diluted to 1:4 with veronal buffer and inactivated at 60°C for 20 minutes. Six serial 2-fold dilutions of each inactivated serum and of each antigen were made in test tubes starting at 1:4. The serum dilutions were distributed on the plates (1 drop per well) using dropping pipettes with blunt 18 G needles calibrated to give 0.025 ml. per drop. One drop of complement (1:30) was added to

each well, and then one drop of the antigen dilutions to the appropriate wells. With each test a serum control, an antigen control and a complement titration were also included. The plates were incubated overnight at 4°C, and the following morning 1 drop of sensitized sheep erythrocytes was added to each well. The plates were gently shaken and incubated for 30 minutes at 37-40°C with occassional shaking. At the end of this time the plates were placed in a refrigerator to allow complete settling, and then read on a scale of 0 (complete hemolysis) to 4+ (no hemolysis).

RESULTS

CROSS-HI TESTS

The results of the cross-HI tests of the Utinga virus with the other members of the Simbu group are shown in Table II. Complete cross-HI testing of all viruses against each other was not possible because of the unavailability of hemagglutinating antigens of all the viruses in the Simbu group. As far as can be ascertained, some of these viruses, including Oropouche, Simbu and Yaba 7 do not produce hemagglutinating antigens. Sango, Ib An 5550, Akabane and Sathuperi do produce HA antigens, but these were not available at the time of testing.

The results in Table II are expressed as the reciprocal of the highest dilution of immune serum or ascitic fluid giving complete inhibition of 4 or 8 units of hemagglutinin. The Utinga immune ascitic-fluid gave a titer of 1:320 and no other immune ascitic fluid showed any inhibition of the Utinga hemagglutinating antigen. The Utinga, Manzanilla and Ingwavuma immune ascitic fluids showed low titer inhibition of some of the other antigens.

CROSS-CF TESTS

The results of the cross complement-fixation tests, done in conjunction with Dr. R. E. Shope, are shown in Table III. For this testing an Utinga immune ascitic

fluid (BE An 84785 AF, Ii) produced by only one intraperitoneal inoculation of virus with adjuvant, and an immune serum (BE An 84784, Ser.3i) produced by three inoculations of virus with adjuvant were used.

The results are expressed as the reciprocal of serum dilution/reciprocal of antigen dilution. The Utinga ascitic fluid (AF) showed a low-titer reaction in the homologous system and no cross reaction with any other antigen. The Utinga hyper-immune serum which was anti-complementary at 1:4 showed a high-titer reaction in the homologous system (512/$\geq$ 256) and with the Oropouche antigen ($\geq$ 128/$\geq$ 256), and much less reaction with several other antigens. On the other hand the Oropouche immune serum reacted at a high titer with its homologous antigen (128/64) but showed a relatively low titered reaction with the Utinga antigen (8/64).

CROSS-NT TESTS

Table IV shows the results of the cross-neutralization tests in mice with the Simbu group viruses. The results are expressed as the log neutralization index. In the homologous systems the Utinga immune AF gave the lowest log neutralization index (1.6). The other Simbu group ascitic fluids neutralized $\geq$ 2.6 log LD_{50} more of their respective homologous viruses than the Utinga virus.

Testing the Utinga ascitic fluid against all the viruses of the Simbu group again showed a log neutralization

index with the homologous virus of 1.6 which was one log or more greater than the other viruses.

RESULTS OF SEROLOGIC SURVEY IN BELEM

In all, 1336 sera were screened by HI testing for antibodies to the Utinga virus. Table V, shows the sources of the sera by genus, species and dates when obtained. Of 885 sera, excluding the 1961 human sera from Belem, only 5 sera showed positive HI reactions at a 1:20 or greater dilution, Table VI. Two of these sera were of insufficient quantity for any further testing, but the other three were subjected to neutralization tests in mice against the Utinga virus (See Neutralization Tests).

Four hundred and fifty-one human sera obtained from residents of Belem during the Oropouche epidemic in 1961, were tested by HI for antibodies to the Utinga virus. Using the antigen which had been prepared at YARU, 18 sera were found giving hemagglution inhibition at 1:20 or greater dilutions. Of these 18, however, all except one (positive at 1:40) failed to inhibit the Utinga antigen which was being used at that time by the Belem laboratory. This latter antigen had been prepared at the Belem laboratory and had a 4-fold higher hemagglutinating titer, giving 4 units of hemagglutinin at a 1:16 dilution (cf. YARU antigen 4 units of hemagglutinin at 1:4 dilution). In these tests the homologous immune serum gave a titer of 1:80 with the YARU antigen, and 1:160 with the Belem antigen.

All the 18 sera were again prepared by the acetone extraction method, and retested by HI with both the YARU and Belem antigens. This time only 6 of the 18 sera reacted at 1:20 with the YARU antigen and none reacted with the Belem antigen. The homologous immune serum reacted at 1:40 and 1:80 respectively (cf. 1:80 and 1:160 in first test).

None of these 18 sera reacted in CF with either Oropouche or Utinga antigen. One of the sloth sera which had reacted at 1:40 in HI was also tested by CF, and did not react with either the Oropouche or Utinga antigens. These results are summarized in Table VII.

NEUTRALIZATION TESTS

Neutralization tests in mice were performed against the Utinga virus with sera from the following sources: (1) Sera obtained in 1961 from residents of Belem who had experienced febrile illnesses during the Oropouche epidemic and from whose blood the virus was isolated. These sera represented the second or third bleedings, about 1 or 2 months after the illness, and all neutralized and/or reacted in CF tests with Oropouche. None reacted in HI with Utinga. (2) Sera from ungulate animals (1 bull, 3 pigs) which in earlier testing by the Belem Laboratory had shown positive HI reactions (at 1:20 or greater) with Utinga antigen. Two of these animals had been brought in from Marajo Island, across the bay from Belem, one from Chaves and one from Porto de Pedro, all areas in close proximity to Belem. (3) Sera from the 2 sloths and 1

marsupial which had reacted in HI tests with Utinga. (4) One serum from a laboratory employee whose serum from 1963 reacted in HI with Utinga. Since the 1963 sample was of insufficient quantity for further testing, a sample obtained 2 years later (1965) was used. This sample did not react in HI.

The results of the neutralization tests are shown in Table VIII. The Utinga immune ascitic fluid gave a log neutralization index ≥ 2.4. Of the 4 human sera only one (Belem 945) showed a suggestion of neutralization with a log neutralization index of 1.6; the other were all < 1.0. The two sloth sera and one pig serum gave log neutralization indeces of 1.2, 1.6 and 1.3 respectively and all the other sera tested were less than 1.0.

DISCUSSION

When the Utinga virus was isolated in 1965 the first step was to determine whether the isolate was an arbovirus. Although the ideal and conclusive evidence for deciding that a virus belongs in this family would be the experimental reproduction of the natural cycle, involving the virus, host, and vector, this is seldom possible. With the Utinga virus this would have been impossible, since as yet no vector had been found for this agent.

Among the considerations which contributed to the inclusion of the Utinga virus with the arbovirus group were the following:

1. The virus was isolated from a lower animal, captured in a tropical rain forest with abundant numbers of arthropods.
2. It was consistently pathogenic for baby mice.
3. It was inactivated by sodium desoxycholate.[43]
4. An agglutinin for goose red blood cells was demonstrated in infected mouse brain.
5. It was serologically related to other viruses of the Simbu group which are currently considered to be arboviruses.

Pathogenicity for mice, sensitivity to sodium desoxycholate, and hemagglutination of goose cells are properties common to many arboviruses. Casals[7] considers that the finding of a serological relationship to an arbovirus

justifies the inclusion of an agent in the arbo "family."

A logical approach to the classification of arboviruses based on antigenic relationship was first formulated by Casals.[5,9] As defined by him,[8] distinct viruses that cross-react by one or several serologic tests are considered as members of a group, with the understanding that the degree of overlap can vary from one extreme, where the viruses are nearly indistinguishable, to the other extreme in which it is difficult to demonstrate any relationship. Viruses are assigned to established groups, or to new groups, on the basis of the reactivity of their homologous sera with other viruses; the reactivity being due to the fact that common antigenic constituents are shared by the members of a group.

Of the serological tests, the HI test with multiple-injection sera (or ascitic fluids) has been used most widely for classification purposes. Multiple injection sera (or AF) are preferred to single-injection sera since it has been repeatedly shown that sera from animals given several injections of one virus react with a far wider spectrum of antigens than do sera produced by a single injection.[6] The basic value of the HI test for classification purposes lies in the fact that, in general, it shows a greater width of overlap than either the CF or NT test, and in the ease with which it can be carried out in large scale. There are cases, however, in which the latter tests are more inclusive. With group C[11] and Bunyamwera[10] viruses, for example, the CF test shows a wider overlap than does the HI.

With the Simbu group it appears that the CF test is also more applicable for grouping purposes. For one thing, many of the members of this group have not been shown to produce hemagglutinating antigens, and secondly, the cross reactivity by HI is less than by CF. By CF tests, on the other hand, there is significant cross-reactivity among several of the agents, and by this method the relationship between the Utinga and Oropouche viruses was first noted. On the basis of Casals'[7] proposed criteria of classifying a new virus in a given group, if it cross-reacts with other members of that group, the Utinga virus may thus be classified in the Simbu group. It should also be re-emphasized that testing of this new agent by the Belem Laboratory against reference group sera of the other viruses of that region failed to show any antigenic similarities.

On the other hand, the absence of, or very low-titer cross reactions of Utinga with the other members of the Simbu group by HI, CF and NT testing indicates that this new agent is indeed a distinct virus and not simply a new strain of one of the other viruses. The results of the cross-NT tests are admittedly not completely clear with regard to the greater neutralizing capacity of the Utinga virus by its homologous serum as compared with the other sera (or AF). This may be due to the lack of success in producing a hyperimmune Utinga ascitic fluid with a high neutralization index. On the other hand, the absence of

cross-neutralization between the Utinga and Oropouche viruses is in agreement with the results previously noted by Bensabath.[3]

Of the 885 human, mammal and bird sera screened for HI antibodies to the Utinga virus only 5 sera (2 sloths, 1 marsupial, 1 rodent, 1 human) showed low-titer positive reactions. The finding of such low-titer reactions is in itself not very conclusive evidence of actual infection by an agent (for reasons that will be discussed below), and it is essential to obtain confirmatory evidence by at least one other serological test, although admittedly this is not always possible.

It is generally accepted that NT antibodies remain detectable for long periods, especially after clinical infection. Less is known about the duration of HI antibodies, but apparently they persist considerably longer than CF antibodies, and in general the results of HI tests usually agree with those of the NT tests.[18]

However, when three HI positive sera were tested by NT tests, the results were at best equivocal. Whereas in that test the homologous hyperimmune ascitic fluid neutralized $\geq$ 2.4 log LD_{50} of virus, the 2 sloth sera showed log neutralization indices of only 1.2 and $\geq$1.6 respectively, and the marsupial serum only 0.5 or less. As inconclusive as these results are, it is interesting that these, at least suggestive reactions, were found in sloth sera; the sloth being the only animal to date from

which the Utinga virus has been isolated. Also, both of these sloths were of the Bradypus tridactilus species. However, it should also be noted that one serum from a pig (UN 43) also gave similar inconclusive results.

Some of the factors that might explain the above results should be considered. It is possible that the positive HI reaction may have been due to non-specific inhibitors which were not removed by the acetone treatment of sera, or that these were expressions of heterologous cross-reactions. Such cross-reactions could be due to one of the other currently known Simbu group viruses, or even to an agent that as yet has not been isolated. It is even possible, although admittedly less likely, that such a cross-reaction could be with agents from a different arbovirus group. Whitman and Shope,[41] and Casals[8] have reported low-grade cross-reactions between arboviruses that hitherto have been considered as belonging to distinct antigenic groups.

If, on the other hand, we assume for the moment that the HI reactions were specific, what could explain the negative, or at best inconclusive, results of the NT tests? It is possible that animals simply do not produce high titers of NT antibody to Utinga infections. This possibility is also suggested by the fact that in the cross-neutralization tests the homologous immune ascitic fluid, produced by several inoculations of virus with adjuvant,

gave only 1.6 logs of neutralization.

Another possibility, which has not been ruled out, is that either the virus is not easily neutralizable, or that "accessory factor" is needed in these reactions. Recently differences have arisen concerning the role of the "accessory factor." It was noted by Sabin[26] that neutralization of the mouse-adapted dengue virus by intracerebral tests in mice was dependent on two factors - a nonspecific, heat-labile accessory substance, and a specific antibody which is heat-stable (56°C for 30 min.). Addition of fresh animal serum, which in itself has no neutralizing activity, fully restored the neutralizing capacity of a heated dengue-immune serum. Similar effect have been noted for vaccinia virus,[20] and Western equine and St. Louis encephalitis immune sera.[19] At the same time it appears that at least in some instances incubation is mandatory, or virtually so, in order to reveal a neutralization effect.[19] In their report on Oropouche virus, Anderson et al[2] noted that neutralization of this virus was also markedly enhanced by the addition of fresh serum, i.e., accessory factor. As previously noted, no accessory factor was employed in the present NT tests with Utinga.

The possibility that NT antibodies for Utinga may decay faster than HI antibodies should also be considered, although this seems less likely. Although it is interesting to speculate about the significance of the above results, it

is my opinion that without further confirmatory evidence these findings cannot be interpreted as indicative of infection with the Utinga virus.

The results of the HI tests with the Belem human sera from 1960-1961 were negative except for a few, low-titered reactions. The significance of these low titered reactions is difficult to interpret. The lack of consistent, reproducible results would seem to indicate that the observed reactions were probably due to non-specific inhibitors in the sera, rather than to specific antibodies. The difference in reactivity of these sera with the Belem and YARU antigens cannot be explained at this time.

As had been noted in the cross-HI, CF, and NT tests the relationship of Utinga virus even with Oropouche is a limited one. It is found only by the CF test, and appears to be a one-way reaction, with the Utinga hyperimmune serum reacting with the Oropouche virus but not vice-versa. Thus, the Belem human sera positive by CF and NT for Oropouche, did not react with the Utinga virus. Here again, some of the factors mentioned, such as use of an accessory factor, may be important in explaining the difference.

It thus appears that at least in the species tested, the Utinga virus does not seem to be widely distributed. The vector responsible for its transmission has not yet been identified, and it is possible that the species which is its main reservoir has not even been tested.

Failure to demonstrate antibodies to this virus in the large number of human febrile cases that were screened suggests that this agent is probably not an important factor in human illness in the Belem area.

SUMMARY

A newly isolated arbovirus, the Utinga virus, was studied to determine some of its serological characteristics and epidemiology. Cross hemagglutination-inhibition, complement-fixation, and neutralization tests established that this virus belongs to the Simbu group of Arboviruses, cross-reacts by CF with the Oropouche virus but is distinct from any of the other members of this group.

A serological survey of 1336 human, mammalian and bird sera from the Belem region of Brazil disclosed only 5 sera with low-titer positive HI reactions with the Utinga antigen, but these could not be confirmed by other serologic tests. The absence of antibodies in the human sera indicates that this agent probably has not been an important factor in human illness in the Belem region.

TABLES

and

FIGURES

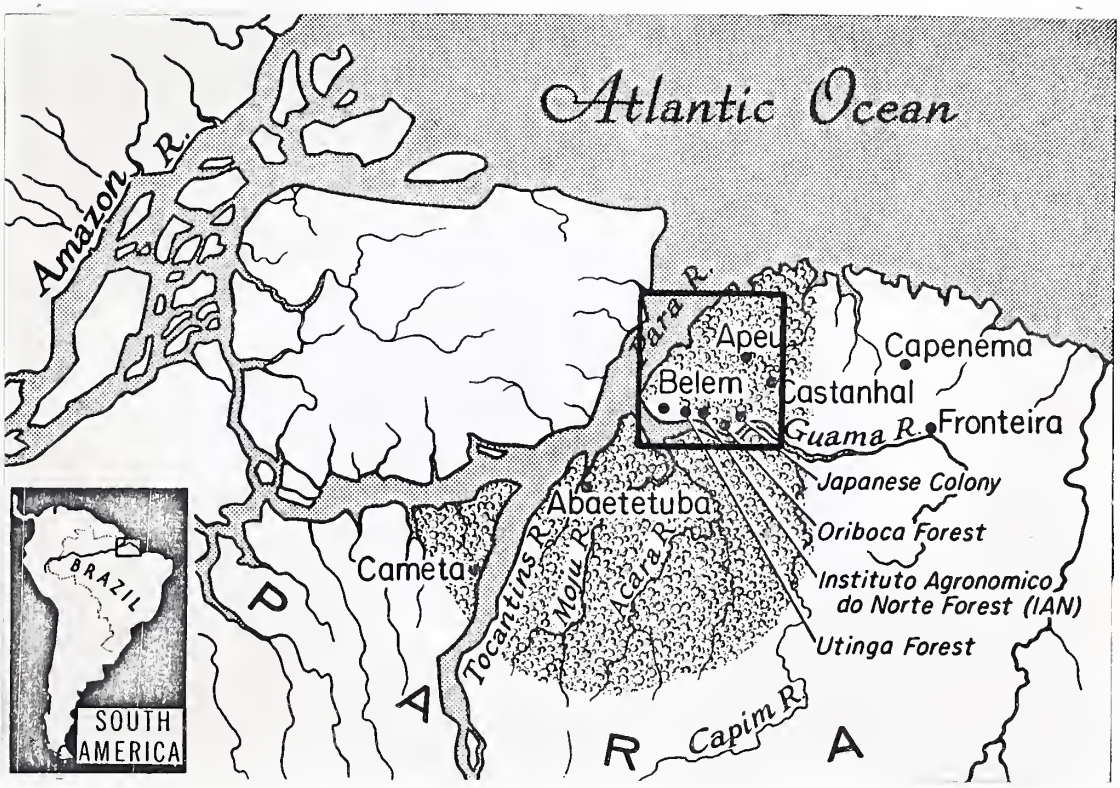


Figure 1. Map showing location of the Belém study areas on the Pará and Guamá Rivers in the State of Pará, Brazil. Area inclosed within square represents region from which all of the sera screened for antibodies to the Utinga Virus were obtained. Shaded area represents tropical rain forest climate zone. (Map after Causey, et al, Am. J. Trop. Med. & Hyg., 10:228, 1961.)

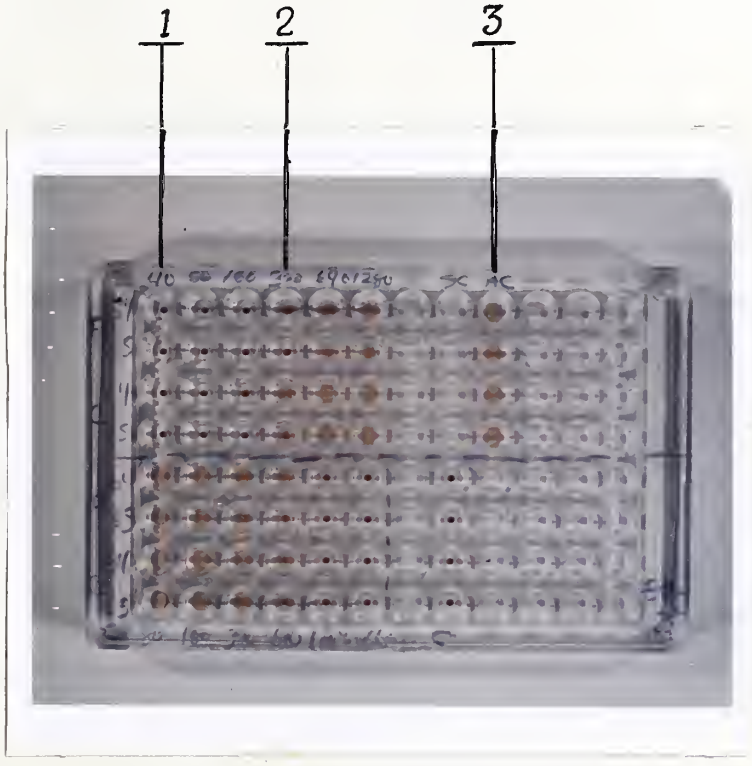
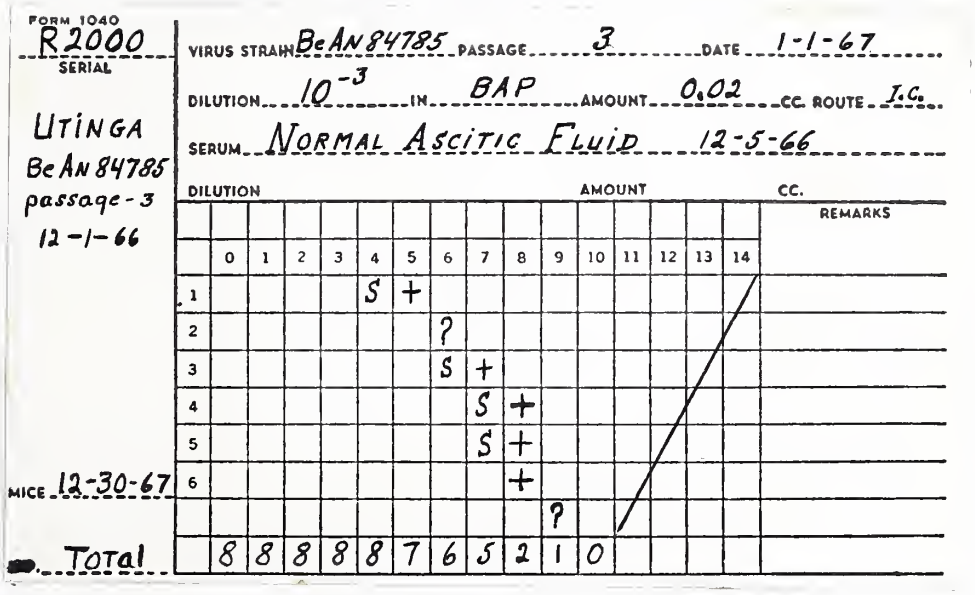


Figure 2. A hypothetical arrangement of an HI test on a clear Lucite plate (Cooke Engineering Company, Alexandria, Va.). The results as shown are read as follows:

1 0 = complete hemagglutination-inhibition
2 ⊖ = partial hemagglutination-inhibition
3 + = no hemagglutination-inhibition



FORM 1040
R2000
SERIAL

UTINGA
BeAN 84785
passage-3
12-1-66

VIRUS STRAIN BeAN 84785 PASSAGE 3 DATE 1-1-67
DILUTION 10^{-3} IN BAP AMOUNT 0.02 CC. ROUTE I.C.
SERUM NORMAL ASCITIC FLUID 12-5-66

DILUTION	AMOUNT CC.														REMARKS
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1					S	+										
2							?									
3							S	+								
4								S	+							
5								S	+							
6									+							
										?						
Total	8	8	8	8	8	7	6	5	2	1	0					

MICE 12-30-67

Figure 3. A typical "mouse card" used for recording data of neutralization tests in mice. The hypothetical data shown is interpreted as follows:

+	=	mouse died
?	=	mouse missing from cage, presumed to have died and been eaten by mother
S	=	mouse showing obvious evidence of illness
Total	=	number of mice surviving on any particular day

TABLE I

THE SIMBU GROUP OF ARBOVIRUSES

Virus	Date of Isolation	Location of First Isolation	Original Source	Hemagglutinin	Human Isolation or Antibody
Akabane[21]	1959	Akabane, Japan	Mosquito, *Aedes vexans nipponii*	Yes	No
Buttonwillow[34]	1961	Kern County, Calif., U.S.A.	*Sylvilagus audubonii*	Yes	No
Ingwavuma[33]	1959	Natal, South Africa	*Hyphanturgus ocularius*	Yes	No
Manzanilla[1]	1954	Trinidad	*Alouatta seniculus insularis*	Yes	Yes (antibody)
Oropouche[2]	1955	Trinidad	Man	No	Yes (isol. and antibody)
Sathuperi[32]	1957	Madras, India	*Culex vishnui*	Yes	No
Simbu[39]	1955	Natal, South Afrida	*Aedes* (N) *circuluteolus*	No	Yes (antibody)
Utinga	1966	Belem, Brazil	*Bradypus tridactylus*	Yes	No
Sango[44]	1965	Ibadan, Nigeria	Bovine	Yes	Not tested
Ib An 5550[44]	1965	Ibadan, Nigeria	Bovine	?	Not tested
Yaba[7]	1963	Lagos, Nigeria	*Mansonia africana*	No	Not tested

TABLE II

CROSS HI TESTS WITH SIMBU GROUP VIRUSES

ANTIGEN	Antigen Units Used	pH	Immune Ascitic Fluids: Utinga	Buttonwillow	Manzanilla	Ingwavuma	Akabane	Oropouche	Sathuperi	Simbu	Sango	Ib An 5550	Yaba 7
Utinga	4	6.5	320	0*	0	0	0	0	0	0	0	0	0
Buttonwillow	4	6.0	20	40	10	10	0	0	0	0	0	0	0
Manzanilla	4	6.1	40	0	40	20	0	0	0	0	0	0	0
Ingwavuma	8	6.0	20	0	0	160	0	0	0	0	0	0	0

*0 = <1:10

TABLE III

RESULTS OF CF TEST OF 3-28-66 WITH SIMBU GROUP VIRUSES

ANTIGENS	Sera or Ascitic Fluids											
	Aka-bane AF 5i	Button-willow AF 2i	Ingwav-uma AF 2i	Manzan-illa AF 2i	Oropou-che Ser. 4i	Sathu-peri AF 5i	Simbu AF 5i	Be An 84785 AF 1i	Be An 84785 Ser.** 3i	Sango AF 2i	Y7 Ser. 4i	Ib An 5550 AF 3i
Akabane	128/256*	0	0	0	0	128/256	64/256	0	4/64	16/256	16/256	≤16
Buttonwillow	0	8/64	16/64	4/4	4/64	4/64	4/16	0	16/64	0	0	0
Ingwavuma	0	0	256/256	16/64	4/64	0	0	0	4/64	0	0	0
Manzanilla	0	0	256/64	32/64	4/16	0	0	0	16/256	0	0	0
Oropouche	4/4	0	8/64	0	128/64	32/64	16/64	0	>128/256	0	0	0
Sathuperi	32/64	0	0	0	0	124/256	128/256	0	16/256	8/64	≤16	64/64
Simbu	64/64	0	0	0	0	>128/64	512/256	0	16/64	8/64	0	4/64
Be An 84785	0	0	4/64	0	8/64	4/16	8/64	16/256	512/256	0	0	0
Ib An 5077	32/64	0	0	0	0	>128/256	64/256	0	8/64	32/256	0	8/64
Y7	128/64	0	0	0	0	256/256	256/256	0	16/256	16/64	128/64	≤16
Ib An 5550	32/16	0	0	0	0	>128/64	128/64	0	16/256	8/64	0	64/64

* Reciprocal of serum/reciprocal of antigen
** Anti-complementary at 1:4

TABLE IV

CROSS-NT TESTS WITH SIMBU GROUP VIRUSES*

VIRUS	Immune Ascitic Fluids										
	Utinga	Akabane	Buttonwillow	Ingwavuma	Manzanilla	Oropouche	Sathuperi	Simbu	Sango	Ib An 5550	Yaba 7
Utinga	1.6	0.6	0.2	1.0	0.6	0.6	0.3	0.0	0.1	0.0	0.2
Akabane	<0.5	5.3									
Buttonwillow	0.5		≥4.7								
Ingwavuma	0.0			≥4.0							
Manzanilla	0.5				≥3.9						
Oropouche	0.6					≥5.2					
Sathuperi	0.0						5.7				
Simbu	0.1							3.1			
Sango	0.0								≥3.7		
Ib An 5550	0.0									2.6	
Yaba 7	0.0										4.7

* Results expressed as log neutralization index.

TABLE V

Sources of Sera Tested for Antibodies to the Utinga Virus

Source	Genus & Species	No. Tested	Years Represented	No. in Each Year
Marsupials		152	1963	34
	Marmosa sp.	40	1965	83
	Marmosa murina	2	1966	35
	Metachirus nudicaudatus	11		
	Caluromys sp.	56		
	Didelphis marsupialis	38		
	Philander opossum	5		
Rodents		219	1965	115
	Oryzomys goeldi	81	1966	104
	Proechimys guyannenis oris	120		
	Nectomys	14		
	Echimys	1		
	Coendou	1		
	Unidentified	2		
Monkeys		91	1963	32
	Cebus	57	1965	44
	Saimiri Sciurus	17	1966	15
	Tamarin Sp	15		
	Howler	2		
Sloths		88		
	Bradypus tridactilus	53	1955-65	-
	Choleopus brasiliensis	11		
	Tamandua sp	3		
	not identified	21		
Bats		21	1963	18
	Unidentified	18	1966	3
	Carollia sp.	1		
	Uroderma bilobatum	1		
	Artibeus cinereus	1		
Birds		171	1966	171
Humans		594	1960-61	451
			1963	48
			1965	69
			1966	26

TABLE VI

Sera Giving Positive HI Reactions
with Utinga Antigen (Total tested - 885 sera)

Serum	Source	Date Obtained	HI titer*
Ed 78	Sloth *Bradypus tridactilus*	5-16-61	40
Bel 212 H 57452	Man	6-24-63	40
Ed 147	Sloth *Bradypus tridactilus*	1-31-65	20
Ma 1909 An 89434	Marsupial *Caluromys sp.*	7-28-65	20
Ro 7242 An 102870	Rodent *Coendon sp.*	6-14-66	20

* HI titer expressed as reciprocal of highest dilution giving complete inhibition.

TABLE VII

Comparison of HI and CF Test Results
with Oropouche and Utinga Antigens

No. of Sera	HI		CF		
			Oropouche		Utinga
	No. Pos. by HI Utinga Ag.* (YARU)	No. Pos. by HI Utinga (Belem)**	No. Pos. in 1961 testing	No. Pos. in 1966 testing	No. Pos. in 1966 testing
18 Human	18 (6 2nd test)	1 (0 2nd test)	0	0	0
8 Human	0	0	8	7	0
20 Human	0	0	0	0	0
1 Sloth	1	Not done	Not done	0	0

* Utinga antigen prepared at YARU
** Utinga antigen prepared at the Belem laboratory

TABLE VIII

Results of NT, CF and HI tests with Selected Sera*

Serum	Source	Date	HI		CF			NT	
			Belem Utinga Antigen	YARU Utinga Antigen	Oropouche Antigen 1961 Testing	Oropouche Antigen 1966 Testing	Utinga YARU ag.	Oropouche 1961 Testing	Utinga 1966 Testing
Utinga Immune Serum	Mice	1966	160	80	-	-	32	-	2.4
Belem 880	Human	1961	0	0	0	0	0	3.0	0.7
Belem 882	Human	1961	0	0	0	8	0	3.5	0.5
Belem 945	Human	1961	0	0	8	16	0	3.5	1.4
Belem 955	Human	1961	0	0	16	16	0	3.5	0.6
Ed 78	Sloth	1961	-	40	-	0	0	-	1.2
Ed 147	Sloth	1965	-	20	-	-	-	-	1.6
MA 1909	Marsupial	1965	-	20	-	-	-	-	0.5
UN 97	Pig	1966	20	-	-	-	-	-	? **
UN 199	Bull	1966	20	-	-	-	-	-	? **
UN 408	Pig	1966	20	-	-	-	-	-	0.7
UN 43	Pig	1966	20	-	-	-	-	-	1.3

* Results are expressed as follows:
HI reciprocal of highest dilution of serum giving complete inhibition of 4 units of hemagglutinin
CF reciprocal of highest dilution of serum giving 3+ or greater fixation
NT log neutralization index

** Technically unsatisfactory

REFERENCES

1. Anderson, C.R., Spence, L.T., Downs, W.G., Aitken,T.H.G, "Manzanilla Virus: A New Virus Isolated from the Blood of a Howler Monkey in Trinidad, W.I." Amer. J. Trop. Med. & Hyg., 9:78-80, 1960.

2. Anderson, C.F., Spence, L., Downs, W.G. and Aitken, T. H.G., Oropouche Virus: A new human disease agent from Trinidad, W.I. Am. J. Trop. Med. & Hyg., 4:574-578, 1961.

3. Bensabath, G., Belem Virus Laboratory, personal communication.

4. Casals, J., Complement-fixation test for diagnosis of human viral encephalitides. J. Immunol., 56:337-341, 1947.

5. Casals, J., Viruses: the versatile parasites, the arthropod-borne group of animal viruses. Trans. N.Y. Acad. Sci., Series II, 19:219-235, 1957.

6. Casals, J., Antigenic classification of arthropod-borne viruses. Proc. VI Internat. Congr. Trop. Med. and Malaria, 5:34-47, 1959.

7. Casals, J., Procedures for Identification of arthropod-borne viruses. Bull. World Health Org., 24:723-734,1961.

8. Casals, J., New developments in the classification of arthropod-borne animal viruses. Proc. VII Internat. Congr. Trop. Med. and Malaria, 11:13-34, 1963.

9. Casals, J., and Brown, L. V., Hemmagglutination with arthropod-borne viruses. J. Exper. Med., 99:429-449, 1954.

10. Casals, J., and Whitman, L., A new antigenic group of arthropod-borne viruses: the Bunyamwera Group. Am. J. Med. and Hyg., 9:73-77, 1960.

11. Casals, J. and Whitman, L., Group C, a new serologic group of hitherto undescribed arthropod-borne viruses, immunological studies. Am. J. Trop. Med. & Hyg., 10: 250-258, 1961

12. Causey, O.R., Causey, C. E., Maroja, O.M. and Macedo, D. G., The isolation of arthropod-borne viruses, including members of two hitherto undescribed serological groups, in the Amazon region of Brazil. Am. J. Trop. Med. & Hyg., 10:227-249, 1961.

13. Causey, C. E. and Causey, O. R., The Arthropod-borne viruses of Brazil in relation to world groups. Revista do Servico Expecial de Saude Publica, 12:9-13, 1962.

14. Causey, O. R., The isolation of virus from natural and sentinel hosts in the Amazon Valley. Revista do Servico Especial de Saude Publica, 12:25-31, 1962.

15. Clarke, D.H. and Casals, J., Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. & Hyg., 7:561-573, 1958.

16. Fulton, F. and Dumbell, K.R., The serological comparison of strains of influenza virus. J. Gen. Microbiol., 3:97-111, 1949.

17. Herrman, E.C., Jr. and Engle, C., Tumor cell-induced mouse ascites fluid as a source of viral antibodies. Proc. Soc. Exp. Biol. & Med., 98:257-259, 1958.

18. Horsfall, F.L. Jr. and Tamm, I.(Ed.), Viral and Rickettsial Infections of Man (4th ed.), J. B. Lippincott Co., Philadelphia, 1965.

19. Lennette, E.H. and Schmidt, N.J.(Ed), Diagnostic Procedures for Viral and Rickettsial Diseases, 3rd Ed., Am. Public Health Assoc., Inc., New York, 1964.

20. McCarthy, K. and Germer, W.D., Two heat-labile factors in normal sera which neutralize variola virus. Brit. J. Exper. Path., 33:529-536, 1952.

21. Oya, A., Okuno, T., Ogata, T., Kobayashi, I. and Matsuyama, T., Akabane, a new virus isolated in Japan. Jap.J. Med. Sci. & Biol., 14:101-108, 1961.

22. Pinheiro, F., Pinheiro, M. Bensabath, G., Causey, O.R. and Shope, R.E., Epidemio de Virus Oropouche em Belem. Revista do Servico Especial de Saude Publica, 12:15-23, 1962.

23. Porterfield, J.S., Use of goose cells in haemagglutination tests with arthropod-borne viruses. Nature, 180:1201-, 1957.

24. Porterfield, J.S. and Rowe, C.E., Hemagglutination with arthropod-borne viruses and its inhibition by certain phospholipids. Virology, 11:765-770, 1960.

25. Reed, L.J. and Muench, H., A simple method of estimating fifty per cent end points. Am. J. Hyg., 27:493-497, 1938.

26. Sabin, A. B., The Dengue group of viruses and its family relationship. Bact. Rev., 14:225-232, 1950.

27. Sartorelli, A.C., Fischer, D.S. and Downs, W.G., Use of Sarcoma 180/TG to prepare hyperimmune ascitic fluid in the mouse. J. Immunol., 96:676-682, 1966.

28. Sever. J. L., Application of a microtechnique to viral serological investigations. J. Immunol., 88:320-329, 1962.

29. Shope, R. E., The use of a micro hemagglutination-inhibition test to follow antibody response after arthropod-borne virus infection in a community of forest animals. Anais de Micro., Vol. 11:167-171, 1963.

30. Shope, R.E., The serological identification of arthropod-borne viruses. Revista do Servico Especial de Saude Publica, 12:33-38, 1962.

31. Simpson, D.I.H. and Williams, M.C., Identification of viruses isolated in Nigeria. East African Virus Research Institute Report - July 1963 to December 1964. Report #14:32-34, 1965.

32. Taylor, R.N.(Ed), Arthropod-Borne Virus Catalogue of the World, Sathuperi #42, U. S. Government Printing Office, 1967, (in press).

33. Ibid., Ingwavuma #126.

34. Ibid., Buttonwillow #140.

35. Theiler, M., Studies on the action of Yellow Fever virus in mice. Am. J. Trop. Med., 24:249-272, 1930.

36. Theiler, M., Action of sodium desoxycholate on arthropod-borne viruses. Proc. Soc. Exper. Biol. & Med., 96: 380-382, 1957.

37. Theiler, M. and Downs, W. G., Oropouche: the story of a new virus. Yale Scientific Mag., March, 37:10, 1963.

38. Tikasingh, E.S., Spence, I. and Downs, W. G., The use of adjuvant and Sarcoma 180 cells in the production of mouse hyperimmune ascitic fluids to arboviruses. Am. J. Trop. Med & Hyg., 15:219-226, 1966.

39. Weinbren, M.P., Heymann, C.S., Kokernot, R.H. and Paterson, H.E., Studies on arthropod-borne viruses of Tongaland. VII. Simbu virus, a hitherto unknown agent isolated from Aedes (Banksinella) circumuluteolus. South African J. Med. Sci., 22:93-102, 1957.

40. Whitman, L. and Casals, J., The Guama Group: a new serological group of hitherto undescribed viruses, immunological studies. Am. J. Trop. Med & Hyg., 10: 259-263, 1966.

41. Whitman, L. and Shope, R.E., The California complex of arthropod-borne viruses and its relationship to the Buyamwera group through Guaroa virus. Am. J. Trop. Med. & Hyg., 11:691-96, 1962.

42. Woodall, J.P., Director, Belem Virus Laboratory, Personal communication.

43. Woodall, J.P., Director, Belem Virus Laboratory, Personal communication.

44. Unpublished results in 1965 Annual Report of University of Ibadan Arbovirus Research Project, p. 35.

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